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The activity of cGMP-dependent protein kinase I α is not directly regulated by oxidation-induced disulfide formation at cysteine 43

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The type I cGMP-dependent protein kinases (PKGs) are key regulators of smooth muscle tone, cardiac hypertrophy, and other physiological processes. The two isoforms PKGI α and PKGI β are thought to have unique functions because of their tissue-specific expression, different cGMP affinities, and isoform-specific protein-protein interactions. Recently, a non-canonical pathway of PKGI α activation has been proposed, in which PKGI α is activated in a cGMP-independent fashion via oxidation of Cys⁴³, resulting in disulfide formation within the PKGI α N-terminal dimerization domain. A “redox-dead” knock-in mouse containing a C43S mutation exhibits phenotypes consistent with decreased PKGI α signaling, but the detailed mechanism of oxidation-induced PKGI α activation is unknown. Therefore, we examined oxidation-induced activation of PKGI α , and in contrast to previous findings, we observed that disulfide formation at Cys⁴³ does not directly activate PKGI α *in vitro* or in intact cells. In transfected cells, phosphorylation of Ras homolog gene family member A (RhoA) and vasodilator-stimulated phosphoprotein was increased in response to 8-CPT-cGMP treatment, but not when disulfide formation in PKGI α was induced by H₂O₂. Using purified enzymes, we found that the Cys⁴³ oxidation had no effect on basal kinase activity or K_m and V_{max} values; however, PKGI α containing the C43S mutation was less responsive to cGMP-induced activation. This reduction in cGMP affinity may in part explain the PKGI α loss-of-function phenotype of the C43S knock-in mouse. In conclusion, disulfide formation at Cys⁴³ does not directly activate PKGI α , and the C43S-mutant PKGI α has a higher K_a for cGMP. Our results highlight that mutant enzymes should be carefully biochemically characterized before making *in vivo* inferences.

The type I cGMP-dependent protein kinases play key roles in regulating vascular tone, intestinal motility, memory formation, and nociception in the spinal cord (1). The kinases are activated by cGMP, and cellular cGMP levels are increased by

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the activity of soluble and particulate guanylate cyclases, which are activated by nitric oxide or small peptides, respectively. Conversely, cellular cGMP levels are decreased by phosphodiesterases. Pharmacological cGMP-elevating agents include nitric-oxide donors (nitroglycerin, nitroprusside), direct guanylate cyclase activators (riociguat), and phosphodiesterase inhibitors (sildenafil, tadalafil). These agents are used clinically to treat cardiac ischemia, systemic and pulmonary hypertension, and erectile dysfunction (2).

The type I PKG gene produces splice variants (PKGI α and PKGI β)² that differ in their first ~100 amino acids (3). The kinases have a similar domain structure, which can be roughly divided into N-terminal regulatory and C-terminal catalytic domains. The regulatory domain contains a number of functional subdomains. At the extreme N termini, leucine/isoleucine zippers (LZs) mediate homodimerization and facilitate binding to specific interacting proteins (4–9). After the LZs, each isoform has a unique autoinhibitory (AI) loop containing an inhibitory sequence that binds within the catalytic cleft and blocks substrate access in the absence of cGMP. The LZ and AI domains differ between PKGI α and PKGI β . Next come two tandem cyclic nucleotide-binding domains (cNBD-A and cNBD-B) that have preferences for binding cGMP over cAMP (~2.4-fold higher for cNBD-A and ~240-fold for cNBD-B (10)). The catalytic domain has an N-terminal ATP-binding lobe and a C-terminal substrate recognition lobe, and the catalytic cleft is located in the crevice between these two lobes. In the canonical PKG activation pathway, cGMP binding to the cNBDs induces a conformational change in the regulatory domain that pulls the AI loop from the catalytic cleft, thereby activating the kinase by allowing substrate access to the catalytic center.

In addition to the canonical activation pathway, PKGI α has been reported to be directly activated by oxidation. In an earlier paper, Landgraf *et al.* (11) showed that metal-induced disulfide bond formation between Cys¹¹⁸–Cys¹⁹⁶ and/or Cys³¹³–Cys⁵¹⁹ increased basal kinase activity to ~70% of the maximum activity that could be achieved with saturating amounts of cGMP.³ More recently, Burgoyne *et al.* (12) demonstrated that disulfide-bond formation at Cys⁴³, which lies at the C-terminal

² The abbreviations used are: PKG, cGMP-dependent protein kinase; LZ, leucine/isoleucine zipper; AI, autoinhibitory; VASP, vasodilator-stimulated phosphoprotein.

³ In this manuscript, we number PKGI α residues starting with the initial methionine.

end of the PKGI α LZ, activated the kinase in a cGMP-independent manner. Although PKG dimer formation is stably mediated by the LZ, Cys⁴³ oxidation and disulfide formation cause the two peptide chains in the dimer to become covalently linked. In addition to direct kinase activation, Cys⁴³ oxidation has been proposed to alter the K_m and V_{max} for substrates and to increase PKGI α binding to specific interacting proteins (12). A knock-in mouse containing a “redox-dead” C43S-mutant PKGI α shows phenotypes consistent with a loss of PKGI α signaling (13–19), and these results have been used to argue for a predominant role for Cys⁴³ oxidation in PKGI α regulation. However, the enzymatic properties of the C43S mutant PKGI α were not thoroughly investigated.

Exactly how Cys⁴³ oxidation activates PKGI α is unknown. To explore the underlying mechanism, we began by comparing the activity of wild-type and C43S-mutant PKGI α under reducing and oxidizing conditions. Surprisingly, and in contrast to previous findings, we found that wild-type PKGI α activity was not directly increased by disulfide formation at Cys⁴³ and that Cys⁴³ oxidation had no effect on substrate phosphorylation. In addition, we found that the C43S mutation caused PKGI α to have a lower sensitivity to cGMP-induced activation.

Results

Oxidation-induced PKGI α dimerization does not lead to increased kinase activity in intact cells

In the original report describing activation of PKGI α by Cys⁴³ oxidation, A10 cells were transfected with wild-type or C43S-mutant PKGI α and treated with H₂O₂, and myosin light chain phosphorylation was examined (12). H₂O₂ treatment caused increased PKGI α disulfide bond formation in wild-type but not C43S-mutant PKGI α , and disulfide bond formation was correlated with decreased myosin light chain phosphorylation, presumably through PKGI α -induced activation of myosin phosphatase (5). Using a similar rationale, we examined whether PKGI α Cys⁴³ oxidation led to increased phosphorylation of known PKGI α substrates in cells. We transfected 293T cells with expression constructs for RhoA together with wild-type or C43S-mutant PKGI α and treated cells with 8-CPT-cGMP or H₂O₂. 8-CPT-cGMP-treated cells showed robust RhoA phosphorylation with both wild-type and C43S-mutant PKGI α (Fig. 1A, top panel, compare lanes 1 and 2 and compare lane 4 and 5). As expected, treatment with H₂O₂ caused a pronounced increase in disulfide bond formation in wild-type but not C43S-mutant PKGI α (shown by gel shift under non-reducing conditions; Fig. 1A, bottom panel, compare lanes 1 and 3 and compare lanes 4 and 6). Unexpectedly, H₂O₂ treatment had no effect on RhoA phosphorylation (Fig. 1A, top panel, compare lanes 1 and 3). Similar results were seen when vasodilator-stimulated phosphoprotein (VASP) phosphorylation was examined. PKG phosphorylates VASP at Ser¹⁵⁷ and Ser²³⁹, and phosphorylation at Ser¹⁵⁷ causes VASP to migrate with a higher apparent molecular weight. Upon Western blotting of transfected cells probed with a VASP Ser(P)²³⁹ antibody, VASP runs as a doublet, and 8-CPT-cGMP treatment causes VASP to completely shift to the upper band, indicating double phosphorylation of Ser¹⁵⁷ and Ser²³⁹ (Fig. 1B,

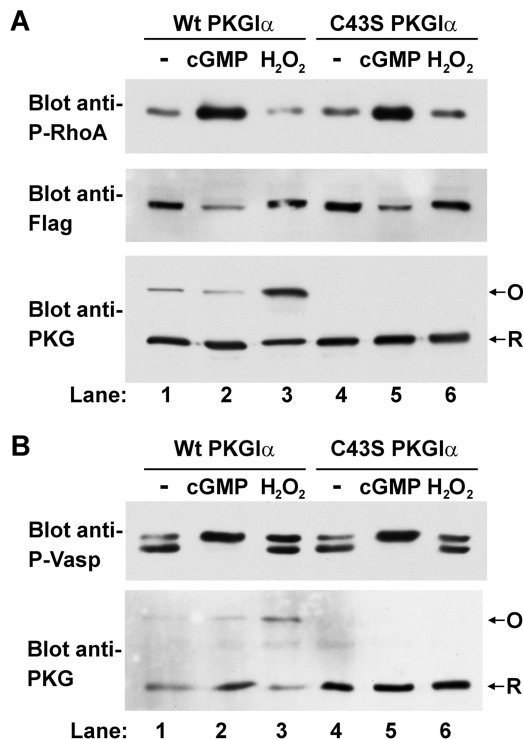


Figure 1. PKGI α is not activated in intact cells by disulfide formation at Cys⁴³. A, 293T cells were transfected with expression vectors for Flag-tagged RhoA (500 ng) and untagged wild-type or C43S-mutant PKGI α (500 ng). The cells were treated for 60 min with 250 μ M 8-CPT-cGMP, 100 μ M H₂O₂, or vehicle alone, as indicated. RhoA Ser¹⁸⁸ phosphorylation was determined by SDS-PAGE under non-reducing conditions followed by immunoblotting with a RhoA Ser(P)¹⁸⁸ specific antibody. The amount of monomeric reduced (R) and disulfide-linked oxidized (O) PKGI α was determined by blotting with a PKGI α specific antibody. B, performed as in A, except 293T cells were transfected with expression vectors for VSV-tagged VASP (200 ng) and wild-type or C43S-mutant PKGI α (200 ng), and blots were probed for VASP Ser(P)²³⁹. These experiments were repeated at least three times with similar results.

compare lanes 1 and 2 and compare lanes 4 and 5). However, in H₂O₂-treated cells, there is only a slight shift in VASP migration, and this shift is seen in cells transfected with wild-type or C43S-mutant PKGI α , indicating that it is not due to Cys⁴³ oxidation (Fig. 1B, compare lanes 1 and 3 and lanes 4 and 6). These results strongly suggest that Cys⁴³ disulfide bond formation does not activate PKGI α in cells.

Oxidation of PKGI α Cys⁴³ does not increase kinase activity *in vitro*

To directly examine whether disulfide formation at Cys⁴³ increases kinase activity, we performed *in vitro* kinase assays. We used anti-Flag affinity beads to purify Flag-tagged PKGI α from transiently transfected 293T cells; this one-step purification protocol allowed us to quickly isolate highly purified full-length wild-type and C43S-mutant PKGI α (Fig. 2A). The purified kinases were incubated for 1 h in 15 mM DTT or allowed to oxidize by exposure to air in the absence of DTT. Immediately prior to measuring kinase activity, aliquots were removed for analysis by non-reducing SDS-PAGE/immunoblotting (as seen in Fig. 2B); under these conditions wild-type PKGI α was ~3.8% oxidized in the presence of DTT and ~59.7% oxidized in the absence of reducing agent. As expected, C43S-mutant PKGI α did not form a disulfide bond and ran as a monomer under both

PKGI α is not activated by Cys⁴³ disulfide bond formation

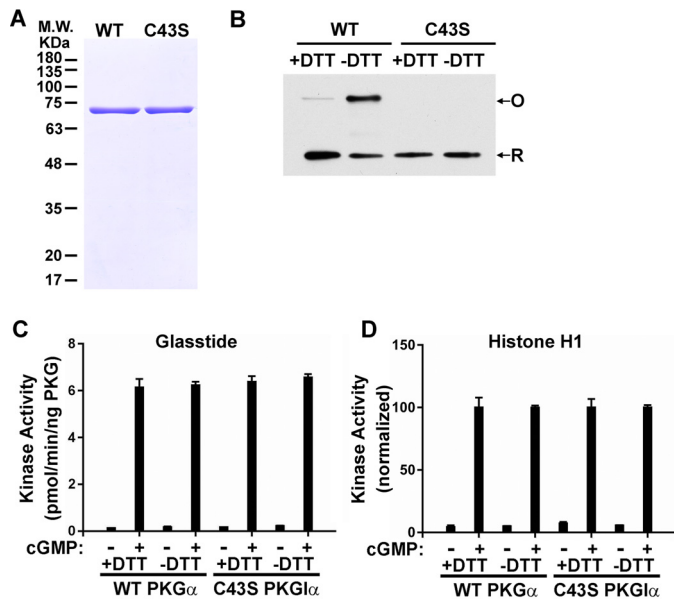


Figure 2. Cys⁴³ oxidation does not activate PKGI α *in vitro*. **A**, Coomassie-stained gel demonstrating the purity and integrity of Flag-tagged wild-type and C43S-mutant PKGI α isolated from transiently transfected 293T cells. **B**, purified wild-type and C43S mutant PKGI α were incubated in the presence or absence of 15 mM DTT and exposed to air for 1 h; the amount of Cys⁴³-cross-linked PKGI α dimer was determined by non-reducing SDS-PAGE/immunoblotting. **C** and **D**, *in vitro* kinase assays were performed in the presence or absence of 10 μ M cGMP using either Glasstide (**C**) or histone H1 (**D**) as substrates. Reactions were performed in triplicate from single protein preparations.

reducing and oxidizing conditions. Kinase assays performed using the small peptide Glasstide as a substrate revealed that oxidation slightly increased the basal activity of both wild-type and C43S-mutant PKGI α (Fig. 2C); basal activity of wild-type PKGI α increased from 0.137 to 0.206 pmol/min/ μ g, whereas activity of the C43S-mutant kinase increased from 0.170 to 0.214 pmol/min/ μ g (maximum kinase activity in the presence of cGMP was \sim 6.2 pmol/min/ μ g under all conditions). Identical results were found using Kemptide as a substrate (supplemental Fig. S1). It was recently reported that PKGI α Cys⁴³ disulfide bond formation increased *in vitro* PKGI α activity toward histone H1 but not RhoA (16). However, in contrast to those results, we found that Cys⁴³ oxidation did not increase PKGI α activity toward histone H1 (Fig. 2D); in fact, we did not even observe the slight oxidation-induced increase in basal activity of wild-type and mutant enzyme seen with small peptide substrates. Thus, under our conditions, oxidation of Cys⁴³ did not directly activate PKGI α *in vitro*.

PKGI α substrate affinity is not altered by oxidation at Cys⁴³

PKGI α Cys⁴³ oxidation has been reported to change the enzyme kinetics (12). In previous studies, Burgoyne *et al.* (12) found that in the absence of cGMP, oxidation decreased the K_m for Glasstide from 247 to 37 μ M but had no apparent effect on V_{max} , whereas in the presence of cGMP, oxidation lowered the K_m for Glasstide from 289 to 89 μ M and lowered V_{max} by \sim 60% compared with the reduced enzyme. Because we found that oxidation did not directly activate PKGI α , we next examined whether oxidation altered PKGI α affinity for peptide substrates. Using purified wild-type and C43S-mutant PKGI α , we

performed *in vitro* kinase assays under the same oxidizing or reducing conditions described above. In the presence of cGMP, we found that oxidation/reduction caused no difference in the K_m or V_{max} for Glasstide in wild-type or C43S-mutant PKGI α (Fig. 3, A and B). In the absence of cGMP, oxidation slightly increased V_{max} in both wild-type and C43S-mutant PKGI α (note the different scales in Fig. 3). This slight increase in basal activity was similar to what was seen in Fig. 2C and was not due to disulfide bond formation at Cys⁴³, because it was the same in wild-type and C43S-mutant enzyme. The complete set of K_m and V_{max} values are listed in Table 1. Fig. 3E shows the amount of reduced versus Cys⁴³-cross-linked PKGI α for each reaction condition.

PKGI α C43S has a reduced affinity for cGMP

We next checked whether oxidation altered the response of PKGI α to cGMP. Using wild-type and C43S-mutant PKGI α under oxidizing and reducing conditions, we performed *in vitro* kinase assays in the presence of increasing amounts of cGMP (Fig. 4). We found that oxidation of wild-type PKGI α had no significant effect on the K_a for cGMP: $K_a = 0.074 \pm 0.003$ and 0.072 ± 0.004 μ M for the reduced and oxidized wild-type enzyme, respectively. However, unexpectedly, we found that the C43S mutation caused PKGI α to require a higher amount of cGMP to half-maximally activate the kinase: $K_a = 0.373 \pm 0.017$ and 0.337 ± 0.015 μ M for the reduced and oxidized mutant enzyme, respectively. Thus, whereas reduction/oxidation had no effect on cGMP-induced activation of wild-type or C43S-mutant PKGI α , the C43S mutation caused PKGI α to be less sensitive to activation by cGMP.

Taken together, our results demonstrate that PKGI α is not directly activated by Cys⁴³ oxidation, and Cys⁴³ oxidation does not change the kinetic properties of the enzyme toward substrates. However, the C43S mutation causes an \sim 5-fold decrease in cGMP affinity of the enzyme. This loss in cGMP sensitivity could explain the reported phenotype of the C43S knock-in mouse, which appears to have a loss of PKGI α function.

Discussion

Since its discovery in the 1970s, PKG has been extensively studied; however, some of its biochemical properties are still incompletely defined. Recent structural studies have demonstrated unique features that play important roles in regulating cGMP-induced kinase activity, including the structural basis for cGMP selectivity and novel interchain contacts that regulate kinase activation (10, 20, 21). Another recently proposed mechanism for PKGI α regulation was direct activation by oxidation-induced disulfide bond formation at Cys⁴³. How the formation of a disulfide bond at Cys⁴³ activates the kinase was not determined, and investigating the mechanism was the starting point for our current study.

In contrast to previous reports, we found that oxidation of PKGI α at Cys⁴³ does not directly activate PKGI α . In addition, Cys⁴³ oxidation had no effect on substrate affinity or reaction velocity using Glasstide as a substrate, and oxidation did not lead to increased histone H1 phosphorylation. Although it is unclear why our results differ from earlier studies, one factor

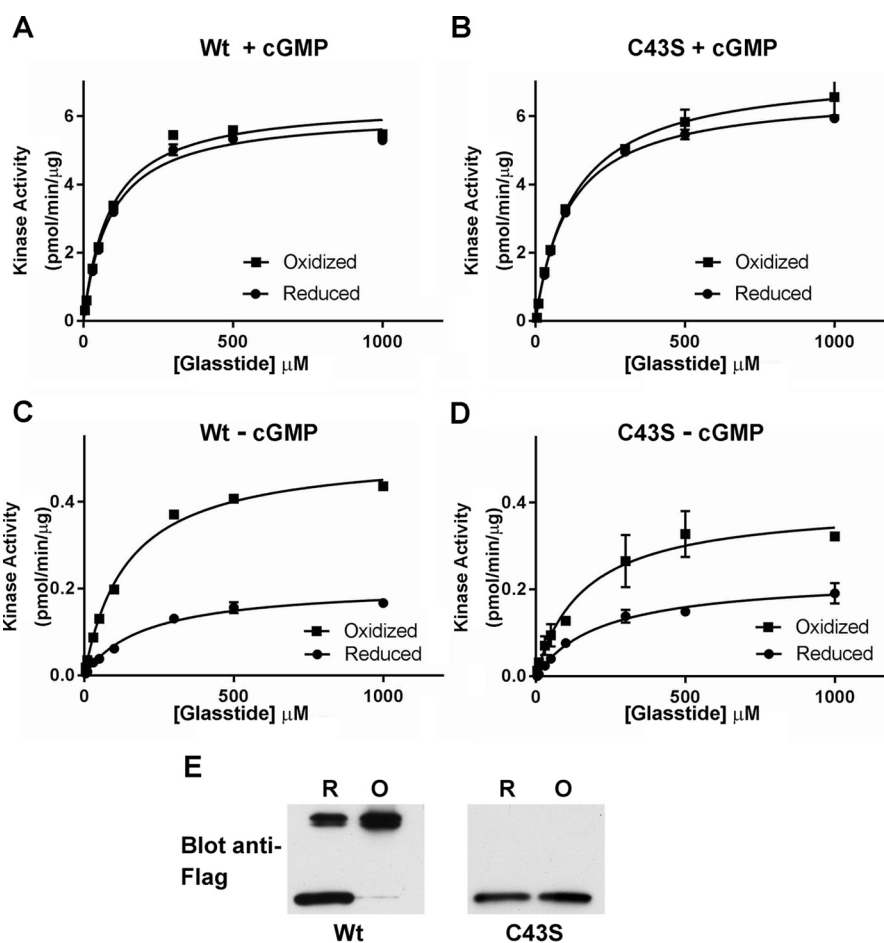


Figure 3. Wild-type and C43S-mutant PKGI α enzyme kinetics. A, PKGI α was incubated in the presence or absence of 15 mM DTT for 1 h, and kinase assays were performed in the presence of 10 μ M cGMP and increasing amounts of Glasstide. B, C43S PKGI α was incubated in the presence or absence of 15 mM DTT for 1 h, and kinase assays were performed as described in A. C, PKGI α activity was measured as in A, but in the absence of cGMP. D, C43S-mutant PKGI α activity was measured as in B, without cGMP. Reactions were performed in triplicate from single protein preparations, and repeated with two independent protein preps. E, Western blot showing relative amount of reduced (R) and Cys⁴³ oxidized (O) PKGI α in the kinase samples used for the reactions. V_{max} and K_m values were calculated using GraphPad Prism 7.

Table 1
Summary of kinetic constants toward Glasstide for wild-type and C43S-mutant PKGI α under oxidizing and reducing conditions

	K_m	V_{max}
	μ M	pmol/min/ μ g
Reduced wild type + cGMP	89.9 \pm 5.5	6.1 \pm 0.1
Oxidized wild type + cGMP	87.8 \pm 3.1	6.4 \pm 0.1
Reduced C43S mutant + cGMP	113.5 \pm 3.9	6.7 \pm 0.1
Oxidized C43S mutant + cGMP	128.3 \pm 8.3	7.3 \pm 0.1
Reduced wild type - cGMP	202.8 \pm 19.5	0.209 \pm 0.0
Oxidized wild type - cGMP	142.7 \pm 7.7	0.514 \pm 0.0
Reduced C43S mutant - cGMP	221.9 \pm 28.9	0.229 \pm 0.0
Oxidized C43S mutant - cGMP	161 \pm 30.5	0.397 \pm 0.0

may be that the original studies were performed using PKGI α purchased from a commercial vendor and that the kinase could only be stimulated 1.5–3-fold by cGMP (12). This suggests that the kinase was proteolytically degraded, leading to a largely cGMP-independent kinase activity, and the poor quality of the enzyme may have affected the biochemical assays. In a subsequent paper by Prysyazhna *et al.* (16), PKGI α was purified using a cAMP-agarose affinity column, followed by cAMP elution and dialysis to remove cAMP. This kinase could be stimulated ~8-fold by cGMP as determined by Western blotting performed with phospho-specific antibodies to detect RhoA and

histone H1 phosphorylation (see Fig. 6A in Ref. 16). In our experience, it is very difficult to fully remove cAMP during PKG purification, especially from PKGI α (10, 20, 22), and in general, Western blotting is semi-quantitative and is not an accurate method to measure kinase activity.

It could be argued that the N-terminal Flag tag on our PKGI α constructs affected kinase activity; however, we note that the kinase used in the present studies was purified intact, had a low basal activity, and could be stimulated 20–50-fold by cGMP. In addition, the Flag-tagged wild-type kinase had a K_a for cGMP of 72–74 μ M, which is consistent with previous results measuring the K_a of untagged PKGI α in cell lysates (23, 24). Our V_{max} and K_m values for Glasstide differ from those obtained by Glass and Krebs ($K_m = 28.8 \mu$ M and $V_{max} = 20$ pmol/min/ μ g (25)); the different values may reflect the variation in kinase assay conditions, because their assays were performed using 2 mM Mg²⁺, whereas we used 10 mM Mg²⁺. In addition, Glass and Krebs had found that for small peptide substrates, PKGI α activity peaked at 2 mM Mg²⁺ and rapidly decreased as free Mg²⁺ levels increased, whereas for large substrates like histone, activity steadily increased as free Mg²⁺ reached 75 mM (26). In comparison with our assays, previous *in vitro* kinase assays determining

PKGI α is not activated by Cys⁴³ disulfide bond formation

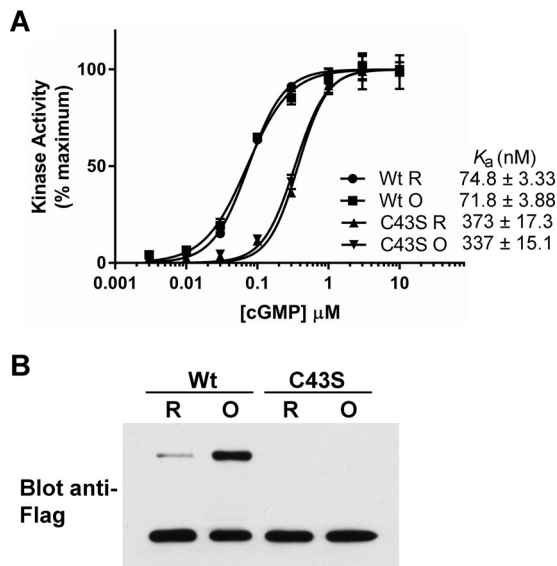


Figure 4. C43S-mutant PKGI α has a decreased affinity for cGMP. *A*, purified Flag-tagged wild-type and C43S-mutant PKGI α were incubated in the presence or absence of 15 mM DTT, exposed to air for 1 h, and used in *in vitro* kinase reactions with 500 μ M Glasstide as a substrate and increasing amounts of cGMP. Numbers are the averages of four values from two independent protein preparations, with reactions performed in duplicate. K_a values were calculated using GraphPad Prism 7. *B*, Western blot showing relative amount of reduced (R) and oxidized (O) PKG in the kinase samples used for the reactions.

the effects of Cys⁴³ oxidation were performed with either 5 or 15 mM MgCl²⁺ (12, 16). We should also point out that the transfected kinases used for our in-cell assays were without epitope tag (Fig. 1), demonstrating that oxidation did not increase the activity of untagged PKGI α in cells. Therefore, we do not feel that the N-terminal Flag tag affected our results.

We found that C43S-mutant PKGI α has an ~5-fold lower affinity for cGMP compared with the wild-type enzyme. This is an important finding, because mice homozygous for C43S-mutant PKGI α have phenotypes consistent with a loss of PKGI α function (17–19). These phenotypes include hypertension, insensitivity to nitroglycerin-induced vasodilation, and protection from septic shock; they have been interpreted to be the result of defective redox-sensing properties normally ascribed to Cys⁴³ disulfide bond formation. However, mice null for the β_1 subunit of soluble guanylate cyclase are also resistant to nitroglycerin-induced vasodilation (27), indicating that the canonical NO-cGMP-PKG pathway is important and that oxidation sensing by Cys⁴³ disulfide formation is not the main mechanism for nitroglycerin-induced vasodilation. Although our current studies have not looked at PKGI α oxidation in mice, our results strongly suggest that defective signaling in PKGI α C43S mice is due at least in part to an increased K_a for cGMP rather than a loss of redox-induced activation of the enzyme.

It is possible that loss of Cys⁴³ oxidation might also affect other aspects of PKGI α signaling; for example, oxidation of PKGI α may alter its interaction with specific interacting proteins, which have been shown to bind to the PKGI α leucine zipper (5, 7, 28). Indeed, oxidation has been shown to increase the *in vitro* interaction between PKGI α and two of its interacting proteins, RhoA and MYPT1 (12). In addition, a recent paper

by Nakamura *et al.* (15) demonstrated that the C43S mutation appeared to alter PKGI α subcellular localization in cardiac myocytes, which suggests a change in association of with interacting/anchoring proteins in cells, but the structural basis for oxidation-induced changes in these interactions was not examined. We are currently pursuing these studies.

How does the C43S mutation lead to decreased cGMP affinity? Since PKGI α and PKGI β were first purified, it has been known that their different N termini cause the two isoforms to have different K_a values for cGMP, even though the sequences of the cyclic-nucleotide-binding pockets are identical (23). We have previously used hydrogen/deuterium-exchange mass spectrometry to study the PKGI β regulatory domain and found that, in the presence of the N-terminal LZ and autoinhibitory subdomains, hydrogen/deuterium exchange was increased throughout the cGMP-binding pockets (29). The increased conformational dynamics correlate with increased cGMP affinity. Because small molecule ligands are thought to stabilize pre-existing protein conformations (30, 31), we reasoned that the N terminus shifted the ensemble of conformations that PKGI β adopts in solution, such that the cyclic nucleotide-binding pockets spend more time in conformations that resemble the cGMP-bound forms. Therefore, it is possible that the C43S mutation, which lies at the end of the LZ, causes a change in the conformational dynamics of the nucleotide-binding pockets, which leads to lower cGMP affinity. In a previous analysis of the PKGI α LZ, the C43S mutation lowered the T_m for thermal denaturation from >108 to 81.4 °C under oxidizing conditions and from 93.0 to 83.3 °C under reducing conditions (32). Although these melting temperatures are not physiological, thermal denaturation measures melting of the entire LZ, and the lower T_m in the C43S-mutant samples may reflect structural destabilization of the region surrounding Cys⁴³, which could occur in the full-length protein under physiological conditions.

In conclusion, we found that disulfide formation at Cys⁴³ does not directly activate PKGI α *in vitro* or in intact cells. In addition, “redox dead” C43S-mutant PKGI α has a higher K_a for cGMP, and this decreased cGMP affinity may at least partially explain the loss-of-function PKGI α phenotype observed in the C43S knock-in mice. Our results also highlight the general fact that mutant enzymes should be carefully characterized biochemically before cellular or physiological inferences are made.

Experimental procedures

Antibodies and reagents

Antibodies specific for Ser(P)¹⁸⁸ of RhoA (sc-32954; lot no. A0914) and PKGI α/β (sc-25429; lot no. F0910) were from Santa Cruz Biotechnology. Anti-VASP Ser(P)²³⁹ antibody was from Cell Signaling Technology (31145; lot no. 5). Anti-Flag M2 antibody F1804; lot no. 101M6216), anti-Flag M2 affinity gel, and Flag peptide were from Sigma. Horseradish-peroxidase-conjugated goat anti-mouse (115-035-062) and goat anti-rabbit (111-035-046) antibodies were from Jackson ImmunoResearch. Kemptide and Glasstide were from AnaSpec, Inc. Histone H1 (sc-221729; lot no. J2115) was from Santa Cruz Biotechnology.

General laboratory reagents were from Fisher Scientific, Sigma Life Science, or Bio-Rad.

DNA constructs

Expression vectors for untagged PKGI α , Flag-RhoA, and VSV-VASP have been described previously (33–35). The Flag-tagged expression vector pFlag-D was constructed by annealing the oligonucleotides 5'-AGCTGCCACCATGGACTACAAA-GACGATGACGACAAGG-3' (sense) and 5'-GATCCCT-TGTCGTCATCGTCTTTGTAGTCCATGGTGG-3' (antisense) and ligating them into HindIII/BamHI-cut pcDNA3. Flag-tagged PKGI α was generated by PCR using the following set of primers: 5'-GACGGATCCGCCGCCATGAGC-GAGCTAGAGGAAG-3' (sense) and 5'-GCACTCGAG-TTATTAGAAGTCTATATCCCATCC-3' (antisense). The PCR product was digested with BamHI/XhoI and ligated into BamHI/XhoI-cut pFlag-D. The C43S mutation in PKGI α was generated using overlapping extension PCR (36, 37), and the mutant PKGI α coding sequence was cut with BamHI/XhoI and ligated into BamHI/XhoI-cut pcDNA3 and pFlag-D to produce untagged and Flag-tagged expression vectors. All PCR-derived PKG constructs were sequenced.

Cell culture and transfection

293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO₂ atmosphere. The cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions (Life Technologies).

RhoA and VASP phosphorylation in 293T cells and Western blotting

293T cells were split into 12-well cluster dishes such that they would be 90–95% confluent 18 h later, at the time of transfection. The cells were transfected with expression vectors for wild-type or C43S-mutant PKGI α and RhoA or VASP as indicated in the figure legends. The next day, the wells were treated for 60 min with 250 μ M 8-CPT-cGMP, 100 μ M H₂O₂, or vehicle as indicated. The medium was aspirated, and the cells were directly lysed by adding non-reducing SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 0.01% bromophenol blue, and 100 mM maleimide (to prevent oxidation during sample processing)). Cell lysates were transferred to microcentrifuge tubes and sonicated 2 \times 20 s at 1-watt power. The proteins were separated by SDS-PAGE, transferred to Immobilon-P, blocked in 5% nonfat dry milk in TBS, and blotted with the indicated antibodies. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Protein expression and purification

The cells were split into a 6-well cluster dish and transfected with Flag-tagged wild-type or C43S PKGI α (three wells each). Approximately 20 h post-transfection, the cells were scraped in PBS and lysed in buffer A (PBS, 0.1% Nonidet P-40) containing 1 \times protease inhibitor mixture (Calbiochem), and lysates were cleared by centrifugation (16,000 \times g, 10 min at 4 °C). Cleared lysates were incubated with 20 μ l of anti-Flag M2 affinity gel (Sigma) for 1 h at 4 °C with constant mixing. The beads were

washed 2 \times 200 μ l of buffer A, 2 \times 200 μ l of PBS with 500 mM NaCl, and 2 \times 200 μ l of PBS. Bound proteins were eluted with 4 \times 10 μ l of elution buffer (PBS with 100 μ g/ml Flag peptide). For each elution step, the beads were incubated with buffer for 5 min on ice. The four eluates for each protein were pooled. Proteins were quantified by SDS-PAGE/Coomassie staining using BSA standards on the same gel. The gels were scanned, and quantification was performed using ImageJ.

Kinase oxidation/reduction and in vitro kinase assays

Flag-tagged wild-type and C43S PKGI α purified from transiently transfected 293T cells were diluted to \sim 1 ng/ μ l in kinase dilution buffer (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, and 0.1% BSA). For reduced samples, the dilution buffer contained 15 mM DTT. 10 μ l of diluted kinase was added to 5 μ l of 3 \times kinase reaction mix (120 mM HEPES, pH 7.4, 1.5 mM Glasstide, 30 mM MgCl₂, 150 μ M ATP, 180 μ Ci/ml [γ -³²P]ATP, and \pm 30 μ M cGMP). The reactions were performed for 1.5 min at 30 °C and stopped by spotting on P81 phosphocellulose paper. Unincorporated [γ -³²P]ATP was removed by washing P81 paper four times for 5 min with 2 liters of 0.452% *o*-phosphoric acid. ³²P incorporation was measured by liquid scintillation counting. In some experiments, 1.5 mM Glasstide was replaced by 1.56 mg/ml Kemptide or 3 μ g of histone H1. The reactions with histone H1 were run for 8 min. For experiments examining enzyme kinetics, reactions were performed \pm 10 μ M cGMP with increasing amounts of Glasstide (0.005–1.0 μ M) or with 1.5 mM Glasstide in the presence of increasing concentrations of cGMP (0.003–10 μ M).

Data analysis

The data were analyzed using GraphPad Prism 7. The V_{\max} and K_m values were measured using non-linear fit Michaelis-Menten analysis, and cGMP K_a values were determined by plotting [agonist] versus normalized response with variable slope.

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